Structural Basis of Rho GTPase Recognition by C3 Exoenzyme

C3 exoenzyme is a mono-ADP-ribosyltransferase (ART) that catalyzes the transfer of an ADP-ribose moiety from NAD$^+$ to RhoA. C3 has long been used to study the diverse regulatory functions of Rho GTPases, however, the substrate recognition and reaction mechanism are poorly understood. We report crystal structures of C3-RhoA complex. These structures reveal that C3 recognizes RhoA via switch I, switch II and interswitch regions. The ARTT-loop of C3 is the key to target recognition. Based on the structural information, we successfully changed Cdc42 to active substrate with combined mutations in the C3-Rho GTPase interface. The structures serve to bridge the gap among independent studies of Rho GTPases and C3.

ADP-ribosylation is an important post-translational protein modification known to be catalyzed by bacterial toxins as well as eukaryotic endogenous ARTs. Based on their target specificity, bacterial ARTs are traditionally classified into several types. C3 exoenzyme and binary toxin enzymatic unit (Ia) ADP-ribosylate RhoA and actin, respectively, however, there is prominent structural similarity between C3 and Ia. Han *et al.* proposed that the bipartite ADP-ribosylating toxin turn-turn (ARTT)-loop, which consists of turn 1 and turn 2, is responsible for substrate recognition, and is thus crucial for the ART activity of C3-like exoenzymes and binary toxins [1]. However, the recognition by ARTT-loop of C3 has never been directly verified, because the structure of a C3-like ART in complex with a protein substrate has never been determined.

We determined the crystal structure of apo (NAD$^+$-free)-C3 (*Bacillus cereus*: C3cer) complexed with human RhoA(GTP). By then soaking the apo-C3-RhoA(GTP) crystal with NADH, we obtained the structure of NADH-bound C3-RhoA(GTP) (**Fig. 1A**) [2]. Using the same approach with C3-RhoA(GTP), we obtained the structure of NADH-C3-RhoA(GDP). C3 recognizes RhoA via switch I, switch II and interswitch regions (**Fig. 1A**). Interestingly, within the structures of both NADH-C3-RhoA(GTP) and NADH-C3-RhoA(GDP) complexes, the switch regions adopted the same conformations as the switch I of GDP conformation and switch II of GTP conformation, respectively. This explains that bacterial C3 exoenzyme ADP-ribosylates both the GTP- and GDP-bound forms of RhoA and utilizes the flexibility of the switch regions of RhoA.

Within the ARTT-loop of C3-like ARTs, (i) a conserved aromatic residue (C3bot1:Phe169/C3cer:Tyr180) in turn 1 has been thought to recognize substrate RhoA via a hydrophobic patch around the acceptor amino acid residue in RhoA (Asn41), and (ii) Gln (C3bot1:Gln172/C3cer:Gln183) in turn 2 has been thought to be essential for interaction with Asn41 (**Fig. 1B**). Actually, within the complex's structure, Tyr180 of C3cer interacts with a hydrophobic patch on RhoA composed of Val43, Ala56 and Trp58, and the hydroxyl group of Tyr180 forms a hydrogen bond with the main-chain carbonyl group of Leu57 (**Fig. 1C**). Importantly, Asn41 formed a hydrogen bond with Gln183 in the QXE-motif within the ARTT-loop of C3cer (**Fig. 1C**). This interaction was conserved in all forms, apo C3-RhoA, NADH-C3-RhoA(GTP), and NADH-C3-RhoA (GDP). This is the first experimental evidence that the glutamine of the QXE-motif directly selects Asn for the ADP-ribosylation.

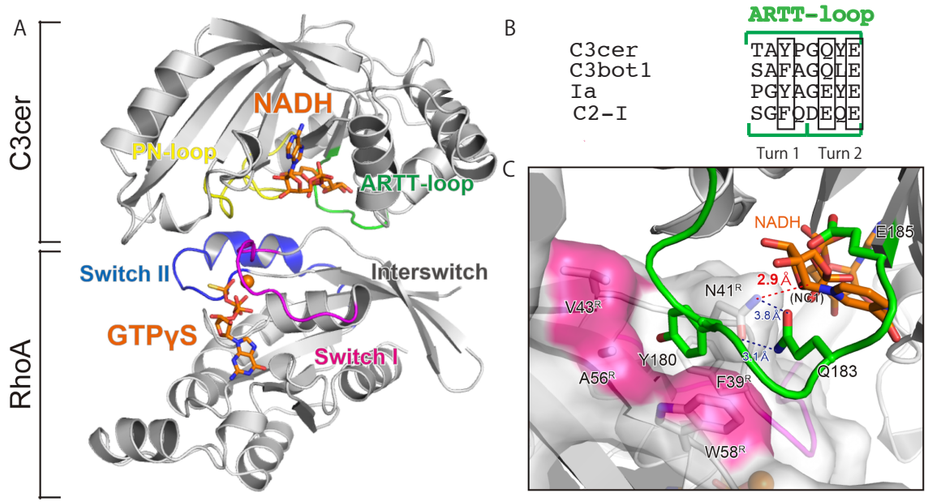

Figure 1: (A) Structure of NADH-C3-RhoA(GTP) complex, **(B)** Sequence alignment of the ARTT-loop of RhoA-specific C3 exoenzymes and actin-specific ARTs (Ia and C2-I), **(C)** Tyr180 on ARTT-loop recognizes the hydrophobic patch of RhoA. Gln183 on ARTT-loop recognizes the modified residue Asn41 of RhoA.

In Ia and C3-like ARTs, a common mechanism has been proposed by which the target protein is recognized using the ARTT-loop: a turn 2 residue (Glu378 in Ia's EXE-motif and Gln183 in C3cer's QXE-motif) interacts with the target residue (Arg177 in actin and Asn41 in RhoA) and an aromatic residue (Tyr375 in Ia and Tyr180 in C3cer) in turn 1 interacts with the region of actin or RhoA (**Fig. 1B**) [1]. However, the recognition by the ARTT-loop has not been observed within the Ia-actin complex [3, 4]. For Ia, the ARTT-loop recognition may occur transiently during the ADP-ribosylation and the transition from the pre- to post-ADP-ribosylation state.

C3-like ARTs modify RhoA but not Cdc42 or Rac1, despite their high amino acid identities. The interaction between the modified Asn41 of RhoA and Gln183 of C3cer is crucial for binding, but Asn41 does not explain the specificity of the C3-RhoA interaction because not only RhoA but also Rac1 and Cdc42 contain asparagine at the same position. Based on the complex structure, we successfully changed Cdc42 to an active substrate of C3cer by introducing four combined mutations (D40E/A43V/T54E/F58W) on switch I and interswitch regions (**Fig. 2**).

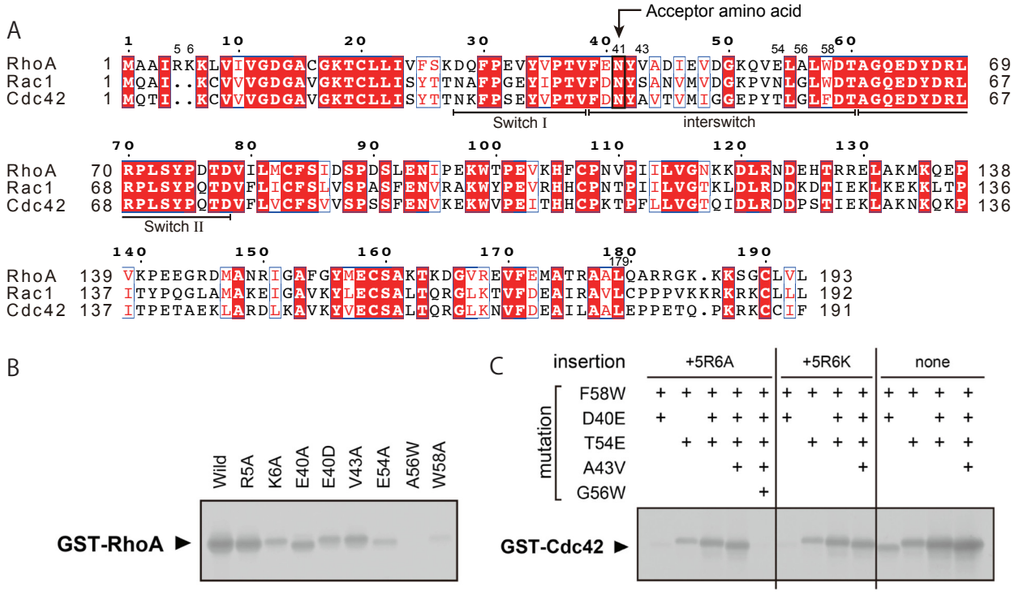

Figure 2: (A) Sequence alignment of RhoA, Rac1 and Cdc42, **(B)** ADP-ribosylation of RhoA single point mutations, **(C)** ADP-ribosylation of Cdc42 combined mutations.

These findings are also useful for understanding the interaction with their substrate of other bacterial ARTs including cholera toxin, mammalian mono-ARTs and mammalian poly-ARTs.

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